OBSERVATIONS ON MICROBIAL FRUCTOSANS AND FRUCTOSANASES[1]

JAKOB R. LOEWENBERG[2] AND ELWYN T. REESE

Abstract

A previously unreported fructosan of *Acetobacter acetigenum* was characterized as similar to that of *Bacillus subtilis-pumilus*, a 2,6-levan. A fructosan of the fungus *Aspergillus sydowi* was characterized as similar to inulin, a 2,1-fructosan. Fructosanases were produced that are specific for either 2,1- or 2,6-linked fructosans. The identification of the fructosans is based on (*a*) the specificity of the fructosanases, (*b*) the rate of movement of fructosan-hydrolyzate products on chromatograms, and (*c*) the presence of detectable amounts of glucose.

Introduction

During the course of an investigation into the mechanism of cellulose synthesis, a fructosan was found in cultures of *Acetobacter acetigenum* grown on sucrose. Members of the genus *Acetobacter* are known to produce cellulose, dextran (4), and starch-like polysaccharides (14) but no reference has been found indicating that they produce fructosans.

The present work describes (*a*) the production of *Acetobacter* fructosan and of a fungal fructosan and (*b*) the production of enzymes that hydrolyze them. A method of characterizing fructosan linkage type (2,1 or 2,6) has been developed which depends upon fructosanases specific for one or the other linkage. Verification of the linkage type is obtained by chromatogramming the oligofructosides derived from the fructosans.

Materials and Methods

Inulin, a 2,1-fructosan found in certain higher plants, was obtained from the Fisher Scientific Company.

Bacillus subtilis-pumilus fructosan was obtained through the courtesy of Professor M. Johnson, University of Wisconsin (11). While this particular preparation (labelled 1624) has not been characterized, *B. subtilis* levans are known to be 2,6-linked (7,1).

Acetobacter acetigenum fructosan was produced from sucrose. Mats of *A. acetigenum* (QM B 1562, NCIB No. 5346 culture originally received from T. K. Walker) were produced by growing the organism in Petri dishes containing 15 ml. of the following medium (per liter): mannitol, 80 g.; sucrose, 20 g.; peptone (Difco), 20 g.; yeast extract (Difco), 20 g.; citric acid.H_2O, 0.83 g.; K_2HPO_4, 1.65 g. The pH of this medium was about 5.2. Cultures were incubated at 30° C. About 4 days after inoculation, a firm mat had formed

[1]Manuscript received February 25, 1957.
Contribution from the Pioneering Research Division, U.S. Army Quartermaster Research and Development Center, Natick, Massachusetts.
[2]Present address: Department of Botany, State College of Washington, Pullman, Washington, U.S.A.

in each dish. These mats were washed well with cold water. Three to six washed mats were placed in cellophane dialysis tubing (5) with about 100 ml. of the following solution (per liter): sucrose, 50 g.; $MgSO_4.7H_2O$, 0.2 g.; citric acid.H_2O, 0.4 g.; K_2HPO_4, 0.72 g.; merthiolate (sodium ethylmercurithiosalicylate), 0.02 g. The dialysis sacs were incubated at room temperature in shallow pans containing an excess of the sucrose solution. The solution in the pans was changed on the third and fifth days. Under these conditions a 12% fructosan solution was produced within the sacs in 2 weeks.

The crude fructosan solution obtained was dialyzed against cold water for several days, filtered through diatomaceous earth, concentrated in a film evaporator (Rotovap) at 40–50° C., and the concentrate poured into nine volumes of acetone. The precipitated fructosan was taken up in a small amount of water and lyophilized.

Aspergillus sydowi (QM 31*c*) fructosan was produced by incubating spores, obtained on potato-dextrose agar, in dialysis sacs containing (per liter): sucrose, 100 g.; KH_2PO_4, 1.36 g.; merthiolate, 0.1 g.; and NaOH in amount sufficient to bring pH of the medium to 5.4. At the end of 1 week a 1.1% fructosan solution was obtained. The solution was dialyzed and added to four volumes of alcohol; the precipitate was taken up in a minimal amount of water and lyophilized.

All four fructosan samples assayed over 100% fructosan on the basis of fructose formed during acid hydrolysis. Ash and "Folin" protein (10) were less than 1% (except for the *Acetobacter* fructosan, which contained about 5% "Folin" protein).

Additional fungi were tested for ability to produce fructosan by incubating spores in the sucrose, phosphate, and merthiolate solution. Fructosan production was accompanied by a change in the appearance of the solution, which became slightly milky.

To test organisms for ability to produce fructosanase they were grown on the two types of fructosans (inulin of 2,1-linkage and *Acetobacter* fructosan, which is shown below to be of 2,6 linkage). The medium contained per liter: yeast extract (Difco), 0.1 g.; KH_2PO_4, 2.0 g.; $(NH_4)_2SO_4$, 1.5 g.; urea, 0.25 g.; $MgSO_4.7\ H_2O$, 0.3 g.; $CaCl_2$, 0.3 g.; fructosan, 4.0 g.; plus the minor elements: Fe, 1 p.p.m.; Zn, 0.8 p.p.m.; Mn, 0.5 p.p.m.; and Co, 0.5 p.p.m. The pH of the medium was adjusted to 6.0 with NaOH. Twenty-five milliliters of medium was used per 125-ml. Erlenmeyer flask. Incubation, usually on a shaker, was at 29° C. At various times during incubation, cell-free filtrates were tested for their ability to hydrolyze fructosans. The fructosanase preparations referred to in this paper are culture filtrates, free of mycelium, to which merthiolate (0.01%) has been added as a preservative, and whose pH has been adjusted to 5.0 with NaOH or citric acid. These preparations contained no reducing sugar or residual fructosans; they did contain other enzymes, some of which are noted below.

Enzyme activities were determined as follows:

(*a*) β-2,1-Fructosanase. To ½ ml. of 0.3% inulin in *M*/20 citrate buffer (pH, 5.4) was added an equal amount of enzyme solution. The mixture was incubated at 50° C. for 1 hour. The reducing sugar liberated was determined as fructose by the dinitrosalicylic acid (DNS) method (13).

(*b*) β-2,6-Fructosanase. As above, using 0.3% *Acetobacter acetigenum* fructosan as substrate.

(*c*) Invertase. As above, using 0.4% sucrose as substrate.

(*d*) Maltase. To 1 ml. of 0.4% maltose, 1 ml. of enzyme solution was added; the mixture was incubated at 50° C. for 1 hour. One milliliter of notatin (glucose oxidase) was added to 1 ml. of the above mixture and shaken at room temperature for 2 hours. Residual maltose was determined by the DNS method.

(*e*) Cellobiase. As for maltase, using 0.4% cellobiose as a substrate.

Fructosan was determined on samples dried overnight at 70° C. after hydrolysis either (*a*) at 100° C. for 30 minutes with 0.5% oxalic acid or (*b*) at 100° C. for 10–60 minutes with *N*/10 HCl. Reducing sugar was determined by the DNS method from a fructose standard curve.

Intermediate products of hydrolysis were obtained by hydrolyzing fructosans (*a*) in 0.33% oxalic acid at 100° C. for 1–3 minutes and (*b*) in fructosanase solutions at 40° C. for 22 hours.

Paper chromatograms were developed for 16–20 hours using isopropanol: acetic acid: water (67:10:23) as solvent. Fructose and fructose containing polymers were detected by spraying the chromatograms with 0.1% resorcinol in 2 *N* HCl (2); glucose was detected with benzidine (8). The rates of movement of compounds are expressed in comparison with the distance moved by fructose, i.e., $R_{fr} = 1.00$ for fructose.

Results

While studying the growth of *Acetobacter acetigenum* (QM B 1562) on various carbon sources, we found a soluble polysaccharide, appearing as a non-mobile fructose-containing spot on chromatograms, when the organism was grown on sucrose. No such material appeared when it was grown on glucose, fructose, glycerol, mannitol, galactose, sorbitol, arabinose, xylose, or sorbose. It was found that non-dividing cells (resting cells) and cell macerates of *A. acetigenum* also produced this polysaccharide from sucrose. Substantial amounts of this material, subsequently identified as a 2,6-fructosan, were produced. *A. xylinum* (QM 1548) also produces fructosan; an acid producer, it gives lower yields and less pure material than *A. acetigenum*. The mechanism of fructosan formation by *Acetobacter* is probably similar to that found in *Aerobacter levanicum* (6).

Since fructosans are poorly known in fungi (as opposed to bacteria), we decided to repeat the work of Kopeloff *et al.* (9) on the *Aspergillus sydowi* fructosan, and to characterize it. Additional fungi were also tested for fructosan production (methods). Three strains of *Aspergillus sydowi* (QM

31*c*, 4*d*, 1990) and three strains of the closely related *A. versicolor* (QM 1989, 4*g* 432) produced fructosan. The poorest among these (QM 1989) produced hülle cells as does *A. nidulans*. The following fungi failed to produce fructosan under the test conditions: *A. nidulans* (QM 1985), *A. silvaticus* (QM 1912), *A. niger* (QM 877), *A. ustus* (QM 6857), *A. janus* (QM 6847) *A. amstelodami* (QM 7091), *Penicillium funiculosum* (QM 474), *Pestalotiopsis, westerdijkii* (QM 381), *Myrothecium verrucaria* (QM 460), and *Trichoderma viride* (QM 6*a*).

Chromatograms of hydrolyzates of the bacterial fructosans showed that the materials contain only fructose. But for a trace of glucose, *Aspergillus* fructosan and inulin also consist entirely of fructose. The glucose that was detected in hydrolyzates of the fungal polysaccharide, and of inulin, was not visible on benzidine-sprayed chromatograms unless these were viewed under ultraviolet light. Bell and Dedonder (1) have also found glucose in inulin, but could not find glucose chromatographically in the bacterial levans they investigated. However, using the glucose oxidase method, they did find traces of glucose in one bacterial levan. It may be that our bacterial levans also contain glucose at this low concentration.

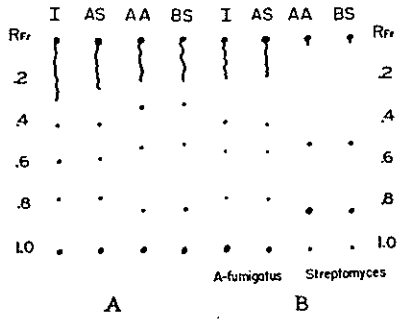

FIG. 1. Hydrolysis products of fructosans; (A) acid hydrolyzates, (B) enzyme hydrolyzates.
I = Inulin (2,1-fructosan)
AS = *Aspergillus sydowi* (QM 31*c*) fructosan
AA = *Acetobacter acetigenum* (QM B 1562) fructosan
BS = *Bacillus subtilis-pumilus* fructosan (2,6-fructosan)

Paper chromatography of the partially hydrolyzed fructosans (Fig. 1) showed that inulin and the *A. sydowi* fructosan are similar and that the two bacterial fructosans are similar. The difructose from the former moves more slowly than the difructose from the bacterial fructosans. From the relationship between log $R_f/(1-R_f)$ and the degree of polymerization (3), it can be shown that acid hydrolysis of inulin and *Aspergillus* fructosan produced fructose, inulobiose, inulotriose, and inulotetrose; and hydrolysis of *Acetobacter* and *Bacillus* fructosans produced fructose, levanbiose, levantriose, and levantetrose. A plot of our data is essentially the same as that of French and Wild (3), even though we used a different solvent system.

To confirm the linkage between the fructose units in *Acetobacter* and in *Aspergillus* fructosans, we attempted to produce enzyme systems that would

specifically hydrolyze either 2,1- or 2,6-linkages; all the fructosans known are of either one or the other type. A total of 50 fungi and bacteria were tested for their ability to utilize fructosans. Like other polysaccharases in fungi, fructosanases are adaptive; that is, little fructosanase (or but a trace) is produced during growth on glucose. Most of the organisms grew well on either inulin or *Acetobacter* fructosan (or both) and so must have the enzymes to utilize them. However, the culture filtrate of most organisms did not contain any detectable fructosanase. Either the fructosanase was not released, or it was rapidly inactivated after release. No fructosanase activity was detected in culture filtrates of the following organisms: *Absidia ramosa* (QM 8*b*); *Aspergillus flavus* (QM 10*e*), *A. luchuensis* series (QM 873), *A. sydowi* (QM 31*c*), *A. unguis* (QM 8*f*); *Basidiomycete* sp. (conidial stage, QM 806); *Botrytis cinerea* (QM 520); *Circinella sydowi* (QM 629); *Chaetomium elatum* (QM 606), *Chaetomium globosum* (QM 459); *Coprinus sclerotigenus* (QM 933); *Monotospora brevis* (QM 1243); *Myrothecium verrucaria* (QM 460); *Paecilomyces varioti* (QM 10*a*); *Penicillium frequentans* (QM 1924), *P. italicum* (QM 6842), *P. lilacinum* (QM 4*e*), *P. notatum* (QM 944), *P. pusillum* (QM 137*g*); *Pestalotiopsis westerdijkii* (QM 381); *Pestalotia virgatula* (QM 478) *Ptychogaster rubescens* (QM 1011); *Pullularia pullulans* (QM 279*c*); *Rhizopus arrhizus* (QM 46*c*) and (QM 1032); *Schizophyllum commune* (QM 812); *Scopulariopsis brevicaulis* (QM 815 and QM 816); *Stachybotrys atra* (QM 94*d*); *Trichoderma viride* (QM 6*a*); *Bacillus megaterium* (QM B1551); *Acetobacter xylinum* (QM B1548), *Acetobacter acetigenum* (QM B1562); *Escherichia coli* (QM B1557); *Pseudomonas aeruginosa* (QM B1468); *Sporocytophaga myxococcoides* (QM B482). Low fructosanase activity, or unstable fructosanase, were found in cultures of: *Aspergillus niger* (QM 458), *A. phoenicis* (QM 1005), *A. terreus* (QM 72*f*); *Bacillus subtilis* (QM B655); *Penicillium commune* (QM 1269); and *Spicaria violacea* (QM 1031).

Active and relatively stable fructosanases were found (Table I) in culture filtrates of: *Aspergillus fumigatus* (QM 45*h*), *Fusarium moniliforme* (QM 527), *Humicola fuscoatra* (QM 34*e*), *Penicillium funiculosum* (QM 474), and *Streptomyces* sp. (QM B814). There is little relationship between the linkage present in the substrate and the enzyme found in the medium. *Fusarium moniliforme* and *Streptomyces* sp. cultures produce the 2,6-fructosanase when grown either on the 2,1- or 2,6-fructosan. Conversely, *A. fumigatus* and *Humicola fuscoatra* cultures yield 2,1-fructosanase on either substrate. As adaptive enzymes, fructosanase formation, it appears, is being stimulated by the fructose split off from the substrate, and not always by the substrate itself. Best activity was obtained in unshaken culture (though it took longer to develop). This suggests that shaking may inactivate the enzymes.

Some of the fructosanases were so unstable that they were completely inactivated at 4° C. in 2–3 days; some lost 50% of their activity in 3 weeks (e.g. the fructosanase of *A. fumigatus*); and some fructosanases were quite stable (e.g. that of *Penicillium funiculosum*). Except for *Streptomyces* sp., maximum fructosanase activity was obtained in unshaken flasks (Table I).

In the exception, peptone was used to stimulate growth. It has been observed before that protein appears to protect enzymes in shaken culture (15).

The culture filtrates contained enzymes in addition to fructosanase (Table II). Invertase activity was high in cultures of *Penicillium funiculosum* and *Fusarium moniliforme*, low in *Streptomyces* sp. and *Aspergillus fumigatus*. Amylase, maltase, and β-glucosidase were found in cell-free filtrates of *Penicillium funiculosum*; and polygalacturonidase in filtrates of *Aspergillus fumigatus*. Cellulase and xylanase were absent in all the filtrates examined.

Culture filtrates were produced that are active on either 2,1- or 2,6-fructosans or on both (Table I). Those active on only one type of linkage totally hydrolyzed that type of fructosan and yet left fructosan of another linkage type virtually intact (Table III). These fructosanase preparations were used to characterize fructosan linkage in much the same way that the structure of α- and β-glucosides is established by their susceptibility to the respective

TABLE I

EFFECT OF SUBSTRATE AND OF SHAKING ON PRODUCTION OF FRUCTOSANASES

Organism	Linkage in substrate for growth*	Maximum potency† (mg. fructose ml.$^{-1}$ hour^{-1})			
		Shaken		Unshaken	
		2,6-ase	2,1-ase	2,6-ase	2,1-ase
Fusarium moniliforme	2,6	0.05	0.01	0.60	0.05
QM 527	2,1	0.10	0.01	0.70	0.0
Streptomyces sp.	2,6	0.25	0.01	0.0	0.0
QM B 814	2,1	0.0	0.0	0.03	0.0
Penicillium funiculosum	2,6	0.26	0.84	1.1	1.22
QM 474	2,1	0.16	0.81	1.02	1.22
Aspergillus fumigatus	2,6	0.05	0.57	0.07	1.16
QM 45 H	2,1	0.0	0.10	0.19	1.22
Humicola fuscoatra	2,6	0.05	0.01	0.07	0.81
QM 34*e*	2,1	0.0	0.05	0.08	0.71

*Growth substrates: inulin = 2,1-fructosan; *Acetobacter* = 2,6-fructosan.
†See "Methods".

TABLE II

ENZYME COMPONENTS OF FRUCTOSANASE AND INVERTASE PREPARATIONS

Preparation	Activity* as hexose (mg. ml.$^{-1}$ 2 hours^{-1})						
	Fructosans				Disaccharides		
	Acetobacter	*Bacillus*	Inulin	*Aspergillus*	Sucrose	Maltose	Cellobiose
Fructosanase							
2,6 of *Streptomyces*	0.34		0.02		0.06		
2,6 of *F. moniliforme*	0.98				1.1	0	0
2,1 of *P. funiculosum*			1.21		0.76	0.40	0.70
2,1 of *A. fumigatus*			0.67		0.07	0	0
Invertase Difco (1/5)	0.09	0.08	0.03	0	1.30†	0.04	0

*Substrate 1.5 mg./ml.; pH 5.4; temperature 50° C.; time 2 hours (except *Streptomyces*, which was incubated only 1 hour).
†The maximum hydrolysis attainable. Dilution to give 50% hydrolysis of sucrose (0.65 mg. hexose ml.$^{-1}$) 1/1200.

glycosidase. Since it is known that inulin is β-2,1-linked, it is highly probable that *Aspergillus sydowi* fructosan, which behaved similarly, has the same linkage. It is also likely that *Acetobacter* fructosan has the same linkage (β-2,6) as the *Bacillus* fructosan.

Enzyme hydrolysis of the fructosans approximated 100% in all cases (Table III). The low value obtained for the *Streptomyces* sp. fructosanase is due to hydrolysis stopping almost completely at the dimer stage. The accumulation of levanbiose here is similar to the accumulation of cellobiose in the *cellulolytic* system of this organism. (We have used *Streptomyces* sp. filtrates to prepare both cellobiose and levanbiose. Each was separated from the other components of the respective hydrolyzates by the carbon column method. The yields were much better than those obtained from *acid* hydrolyzates. About 40% of the original substrate could be recovered as the difructose, levanbiose.)

When some fructosanase preparations acted on their respective substrates, no intermediates (between fructosan and fructose) were detectable chromatographically. The 2,6-fructosanases of *Fusarium moniliforme* and of *Penicillium funiculosum* and the 2,1-fructosanase of *P. funiculosum* behaved in this way. In other systems, intermediates were found (Fig. 1); these were similar to those found in acid hydrolyzates. The accumulation of intermediates is attributed to a relatively low content of oligofructosidases in the fructosanase solutions.

When culture filtrates were incubated with 7% sucrose and chromatogrammed, a slow moving fructose-containing spot (R_f 0.50) and fructose were found in addition to sucrose. It is also of interest to note that commercial invertase preparations (Difco, 1/50) hydrolyzed the dimer of both the 2,1- and 2,6-series. The trimer, tetramer, and even the pentamer of both series were hydrolyzed but more slowly; yet the invertase solution had no appreciable action on either the 2,1- or the 2,6-fructosans (Table II).

Schlubach and Sinh (12) have characterized fructosans on the basis of time required to reach 50% hydrolysis in normal sulphuric acid at 20° C. In our tests, inulin, *Aspergillus* fructosan, *Bacillus* fructosan, and *Acetobacter* fructosan showed no appreciable differences; all four reached the 50% level in 157–160 minutes.

TABLE III

EXTENT OF HYDROLYSIS OF FRUCTOSANS BY ENZYMES

Fructosanase of:	Hydrolysis, %* of fructosan of:			
	Inulin	*Aspergillus*	*Acetobacter*	*Bacillus*
Penicillium funiculosum QM 474	102	113	97	98
Aspergillus fumigatus QM 45H	100	105	4	3
Humicola fuscoatra QM 34e	98	102	10	10
Fusarium moniliforme QM 527	0	0	88	82
Streptomyces sp. QM B814	0	0	50	52

*20 hours, 40° C., pH 5.4.

Acknowledgments

The cultures employed were supplied from the QM Culture Collection by Miss Dorothy Fennell. We appreciate also the information supplied to us by S. A. Barker and M. Stacey on the infrared analysis of our fructosans.

Addendum

Infrared spectra of fructosans. Report of S. A. Barker and M. Stacey (Univ. Birmingham) on our fructosans. Letter of 12/21/56.

(1) Fructosan of *Acetobacter acetigenum* (2:6 linked). "If this is indeed a polyfructose, then the peaks at 918 cm.$^{-1}$ and 800 cm.$^{-1}$ agree well with those we found at 919 cm.$^{-1}$ and 803 cm.$^{-1}$ with a levan from *Bacillus mesentericus*. The appearance of a peak at 1684 cm.$^{-1}$ would not be expected normally of a levan and suggests an impurity in the polysaccharide."

(2) Fructosan of *Aspergillus sydowi* (2:1 linked). "The peaks at 937 cm.$^{-1}$, 868 cm.$^{-1}$, and 810 cm.$^{-1}$ compare with those at 928 cm.$^{-1}$, 870 cm.$^{-1}$, and 812 cm.$^{-1}$ we found with an authentic specimen of inulin. Your extra peak at 833 cm.$^{-1}$ may be due to impurities or the presence of a proportion of α glucose residues in your polyfructosan." "You will see that these [results] confirm, in a rather nice way, your own work."

References

1. Bell, D. J. and Dedonder, R. A structural re-examination of the levans formed by *Pseudomonas prunicola*, Wormald and *Bacillus subtilis* BG 2 F 1. J. Chem. Soc. 2866–2870 (1954).
2. Forsyth, W. G. C. Color reagents for the paper chromatography of sugars. Nature, **161**, 239–240 (1948).
3. French, D. and Wild, G. M. Correlation of carbohydrate structure with papergram mobility. J. Am. Chem. Soc. **75**, 2612–2616 (1953).
4. Hehre, E. G. and Hamilton, D. M. Bacterial conversion of dextrin into a polysaccharide with the serological properties of dextran. Proc. Soc. Exptl. Biol. Med. **71**, 336–339 (1949).
5. Hestrin, S., Avineri-Shapiro, S., and Aschner, M. Enzymic production of levan. Biochem. J. **37**, 450–456 (1943).
6. Hestrin, S., Feingold, S. D., and Avigad, G. The mechanism of polysaccharide production from sucrose. 3. Donor-acceptor specificity of levansucrase from *Aerobacter levanicum*. Biochem. J. **64**, 340–351 (1956).
7. Hibbert, H. and Brauns, F. Structure of the levan synthesized by the action of *Bacillus subtilis* on sucrose. Can. J. Research, **4**, 596–604 (1931).
8. Horrocks, R. H. Paper partition chromatography of reducing sugars with benzidine as a spraying reagent. Nature, **162**, 164–444 (1949).
9. Kopeloff, N., Kopeloff, L., and Welcome, C. J. Formation of gum levan by mold spores. J. Biol. Chem. **43**, 171–187 (1920).
10. Lowry, O., Rosebrough, N., Farr, A. L., and Randall, R. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–277 (1951).
11. Mattoon, J. R., Holmlund, C. E., Schepartz, S. A., Vavra, J. J., and Johnson, M. J. Bacterial levans of intermediate molecular weight. Appl. Microbiol. **3**, 321–333 (1955).
12. Schlubach, H. H. and Sinh, O. K. Untersuchungen uber fructose anhydride. XXIV Die Gruppe der naturlichen Polyfructosane. Ann. **544**, 111–116 (1940).
13. Sumner, J. B. and Somers, G. F. Laboratory experiments in biological chemistry. Academic Press, Inc., New York. 1944.
14. Tosic, J. and Walker, T. K. *Acetobacter acidum-mucosum* Tosic and Walker n.sp. An organism forming starch-like polysaccharide. J. Gen. Microbiol. **4**, 192–197 (1950).
15. Whitaker, D. R. Effect of proteins and proteoses on cellulase of *Myrothecium verrucaria*. Science, **116**, 90–92 (1952).